**PHOSPHATASE TEST - CHARM PASLITE - ALKALINE PHOSPHATASE TEST USING CHARM II 6000/6600 AND LUMINOMETER/LUMINATOR**

**[Unless otherwise stated all tolerances are ±5%]**

**SAMPLES**

**1. Laboratory Requirements (see CP, items 33 & 34)** _____

**APPARATUS**

**2. CP, items 1 - 32 (as necessary)** _____

a. Unless otherwise stated, "shake vigorously" refers to standard microbiological mixing, i.e., 25 times in a one foot arc in seven seconds _____

**3. Incubator Block for 13 x 100 mm test tubes or 2 mL microtubes** _____

a. Thermostatically-controlled at 35±1C _____

b. Temperature checked by electronic display or by thermometer in small well in block or by liquid immersion, records maintained _____

**4. Pipettors and Pipets** _____

a. Fixed volume or electronic, 100 µL _____

b. Calibration checked as specified in CP item 6e, records maintained _____

c. Disposable, 10 mL (ASTM) pipet with 0.1 mL graduations _____

**5. Reagent Dispenser** _____

a. Fixed volume or electronic, 1.0 mL _____

b. Calibration checked (CP item 6e) with 10 mL ASTM graduated cylinder, records maintained _____

**6. Test tubes or microtubes** _____

a. Test tubes for Charm II 6600/Charm II 6000 systems, disposable borosilicate glass 13 x 100 mm, dirt and scratch free _____

b. Microtubes - for Luminometer/Luminator, 2 mL screw cap _____

**7. 6000/6600 or Luminometer/Luminator Analyzer** _____

a. Operating instructions available _____

b. Definitions: _____

1. Fluid white milks - including skim through whole fat milk _____

2. Unflavored liquid dairy products - including half and half, buttermilk, creams (light, medium and whipping), and etc _____

3. Flavored liquid dairy products - Liquid products that can be accurately pipetted, containing flavor additives and/or thickening agents including flavored milks, and etc _____

4. Solid/semisolid dairy products - thick dairy products not able to be pipetted, solid and/ or powdered additives, including cheese, yogurt, cottage cheese, ice cream mix, ice cream milk, whey, sour cream, and heavy cream Ó 36%, and etc _____

**8. Water Bath, circulating, 34±1C and 63±1C (or 66±1C if fat > 10%), or 13 x 100 test tube dry well heater blocks acceptable (Confirmation procedure)** _____

**9. Centrifuge - Charm II Heraeus (3,400 RPM), minifuge, or equivalent (1,200 - 2,000 g)** _____

**10. Handling and storage** _____

a. Kit contains Reagent AP, Stopping Solution and Alkaline Phosphatase Positive Control Tablet _____

Kit: Lot # _____ Rcd. Date _____ Exp Date _____ _____

1. For solid/semisolid dairy products, Diluent AP _____

Diluent AP: Lot # _____ Exp. Date _____ _____

b. Reagents stored at 0-4.4C until expiration date _____

c. Stopping Solution must be at 18-24C at time of use, may be stored at room temperature, expiration date marked 2 month from room temperature storage _____

d. Bottles labeled with receive and open dates _____

## CONTROLS

**11. Negative Control** _____

a. Product type. Prepare at least 50 mL for calibrator and positive control preparation _____

1. Fluid white milk - heat a sample of product (highest fat content) to 95±1C for 1 minute with stirring _____

2. Flavored liquid dairy products - heat a chocolate (highest fat content) to 95±1C for 1 minute with stirring _____

3. Unflavored liquid dairy products - heat pasteurized light cream to 95±1C for 1 minute with stirring _____

4. Solid/semisolid dairy products - mix or knead 5 g of product (highest fat content) with 20 mL Diluent AP until homogeneous and heat to 95±1C for 1 min with stirring _____

5. Note, if using 13 x 100 test tube dry well heater block at 95C it takes 10 minutes to heat product to 95C for one minute, use temperature control _____

b. Cool rapidly in an ice bath and hold at 0-4.4C, _____

c. Kept at 0-4.4C, the Negative Control may be used for up to 48 hours _____

d. If desired, distribute 1 mL quantities in small tubes (Milk only),seal and freeze in a non-frost-free freezer, or place in a styrofoam container and place in the center of a frost-free freezer for no more than 2 months, vials labeled with preparation and expiration dates _____

**12. Positive Control (for daily checks)** _____

a. Reconstitute positive control (450 mU/L) with negative control, item 11, as indicated on label, or alternatively use 350 mU/L calibrator (item 13a2a) _____

b. Shake vigorously and let settle 10 minutes at 0-4.4C for re-suspension _____

1. For solid/semisolid dairy products only, add 1 mL of reconstituted positive control with 3 mL of Negative control to complete preparation of positive control

c. Shake vigorously again and use for test _____

d. Positive controls and calibrators held at 0-4.4C may be used for 48 hours, milk controls may be frozen at -15C or lower for up to 3 weeks, thaw in refrigerator prior to use _____

e. With 6600 and C2Soft, enter either the triplicate RLU average of Positive Control or triplicate RLU average of 350 mU/L calibrator as the pos avg and CP in C2Soft configuration file. Refer to C2Soft manual _____

**CALIBRATION**

**13. With each new kit lot # check calibration of analyzer** _____

a. Prepare 350mU/L, 175mU/L, 44mU/L (milk only), 88mU/L (flavored and unflavored only) calibrators using Negative Control, item 11 _____

1. Rehydrate a calibrator tablet with 100 uL water, mix to disperse tablet, wait 1 minute and mix again _____

2. Add the specified volume of Negative Control to each dissolved calibrator tablet to make calibators:

a. Add 2.5 mL to make 350mU/L
b. Add 5 mL to make 175 mU/L
c. Add 10 mL to make 88 mU/L (flavored and unflavored only)
d. Add 20 mL to make 44 mU/L (fluid white milk only)

b. Calibrate instrument by testing each calibration control (350, 175, 44 (or 88) mU/L) in triplicate _____

**6600 with C2Soft Software**

c. For fluid white milks, unflavored or flavored liquid dairy product on the 6600 system with C2Soft software, follow the Standard Curve Calibration procedure _____

1. Program has a separate assay line for each product group, fluid white milk, flavored and unflavored liquid dairy product _____

2. In calibrate mode, enter low concentration (44 or 88 mU/L) value, followed by 3 replicate counts _____

3. Enter medium concentration (175 mU/L) value, followed by 3 replicate counts _____

4. Enter high concentration (350 mU/L) value, followed by 3 replicate counts _____

5. Calibration successful will be prompted at end of the procedure _____

d. For solid/semisolid dairy products using the 6600 system with C2Soft, follow instructions for positive average or control point setup _____

1. Count 3 replicates of 350 mU/L control _____

2. Control point is equal to average of triplicate counts _____

**Luminometer/Luminator system**

g. For fluid white milk, unflavored or flavored liquid dairy products, determine average value for each calibrator _____

1. Set up a separate channel and calibration for each product group, fluid white milk, flavored and flavored liquid dairy products _____

2. Check calibration _____

a. Average negative control tested in triplicate. Average must be less than 5 (less than 15 for flavored dairy products) _____

b. Average 44 mU/L (or 88 mU/L unflavored and flavored liquid dairy products) calibrator, must be between 35 - 53 mU/L (45 – 110 mU/L unflavored and flavored liquid dairy products) _____

c. Average 175 mU/L positive control, must be 145 - 205 mU/L _____

d. Average 350 mU/L calibrator, must be 320 - 400 mU/L _____

3. If conditions are not met, recalibrate according to Luminometer/Luminator calibration instructions _____

h. For solid/semisolid dairy products verify control point of 350 mU/kg _____

1. Count 3 replicates of 350 mU/kg control _____

2. Average 350 mU/kg positive control, must test 350±105 mU/kg _____

3. If conditions are not met, recalibrate according to Luminometer/Luminator calibration instructions _____

**DAILY PERFORMANCE CHECKS**

**14. Daily and to verify calibration, test a Negative Control (item 11) and Positive Control (item 12), for at least one product** _____

a. Test negative control beginning from item 15a or with solid and semi-solid dairy products from 15a2 _____

b. Verify Negative Control calibration _____

1. Fluid white milk test, unflavored and flavored assay value < 5 mU/L with luminometer or < 44 mU/L (<88 mU/L flavored and unflavored) with 6600 and C2Soft _____

2. Solid and semi-solid dairy products < 350 mU/kg or less than or equal to the control point _____

c. Verify Positive Control calibration _____

1. Positive Control (450 mU/L) rehydrated with fluid white milk, flavored and unflavored fluid dairy products, must be 300-585 mU/L or 350mU/L calibrator must be 247-453 mU/L _____

2. Solid and semi-solid dairy products, within ± 30% of 350 mU/kg or the control point _____

**TEST PROCEDURE**

**15. Procedure** _____

a. Prepare sample _____

1. For fluid white milks, unflavored and flavored, invert filled retail container 25 times, each inversion a full cycle down and up, shake or vortex negative control _____

2. For solid/semisolid dairy products **(not including controls, item 11b & 12)** add 1 part to 4 parts Diluent AP _____

   a. Mix or knead until homogeneous _____

   b. Centrifuge for 3 minutes _____

   c. Use liquid extract in item 15c _____

b. Dispense 100 µL of Reagent AP into test tubes or microtubes _____

c. Dispense 100 µL of the prepared sample (item 15a) or mixed controls (items 11d & 12) just above the Reagent AP and immediately mix _____

   1. Use a new pipet tip for each sample, place pipet tip in sample or prepared control (no more than 1 cm), draw up and remove tip from sample/control, expel once to pre-wet tip _____

   2. Draw sample or control into pipet tip, touch off to side of container _____

   3. Holding pipet 90∀ to lab bench at eye level, dry exterior of tip (if necessary) by wiping from the pipet toward the tip, be careful not to touch end of tip _____

   4. Dispel 100 µL sample directly above surface of Reagent AP (do not dispense down side of test tube or microtube) _____

   5. Depress plunger several times to completely expel sample _____

   6. Mix test tubes or microtubes with a back-and-forth motion for 10 seconds, use a vortex mixer if available _____

d. Place the test tube/microtube in the 35±1C incubator for 3 minutes _____

e. Within 10 seconds after incubation add 1 mL of room temperature (18-24C) Stopping Solution _____

f. Remove test tubes/microtubes from incubator, cap and shake each vigorously or vortex for 10 seconds _____

g. Place test tube/microtube into analyzer within 3 minutes, tubes held at room temperature (Note: stability of count may be stabilized by placing tubes/microtubes in a room temperature bath) _____

1. **6600 with C2Soft software** _____

a. Select appropriate assay type _____

b. Enter ID of sample and press enter _____

c. Load sample in analyzer and press enter _____

d. In 5 seconds RLU reading will be displayed, mU/L value will appear in results or pop-up window _____

e. For solid/semisolid dairy products, sample RLU will be compared to control point _____

2. **Luminometer/Luminator** _____

a. Select AP Assay (or customized channel) _____

b. Press Start or Enter _____

c. In 5 seconds mU/L reading will be displayed _____

h. Counting of all test tubes/microtubes must be completed in 3 minutes _____

i. Samples with Ó 350 mU/L (category 4) (or for solid/ semisolid dairy products, values greater than or equal to control point) of ALP activity are suspect positive containing about 0.1% (v/v) or more raw milk and must be tested for microbial, and reactivated phosphatase (items 16 & 17) _____

**CONFIRMATION**

**16. Microbial Phosphatase** _____

a. Heat 1.0 mL of suspect sample at 63±1C for 30 minutes, stirring or mixing every 10 minutes _____

1. For semisolid/solid dairy products dilute 1.0 g suspect sample with 4.0 mL diluent AP, mix or knead until homogeneous _____

2. If fat content is > 10%, heat at 66±1C for 30 minutes _____

b. Cool sample rapidly to 0-4.4C in an ice bath _____

c. Test positive and negative controls following item 15 _____

d. Test heated sample and unheated sample (original sample) following item 15 (semisolid/solid dairy products begin at item 15b) _____

e. Interpretation _____

1. Controls test as specified in item 14 _____

2. If heated and unheated sample have equal activity (±30%, mU/L or RLU) the sample is regarded **Not Found** for residual phosphatase, the activity originally measured is microbial _____

3. If the heated sample is more than 30% below unheated sample (mU/L or RLU), the sample contains milk phosphatase activity, either residual or reactivated _____

**17. Reactivated Phosphatase** _____

a. Magnesium acetate solution commercially available _____

b. Or, prepared in laboratory _____

1. Dissolve 35.4 g of magnesium acetate tetra-hydrate, $Mg(C_2H_3O_2)_2 \cdot 4H_2O$ in 25 mL MS water, warming slightly to aid dissolution _____

2. Pour solution into 100 mL volumetric flask, rinse original container several times and add rinses to flask _____

3. After cooling to room temperature, make up to 100 mL (stable for 1 year at 0-4.4C) _____

c. Procedure _____

1. Label separate test tubes as "Blank" and "Test" _____

2. Add a 5.0 mL aliquot of sample (unheated, original sample not prepared as in 15a) to each test tube _____

a. For semisolid/solid dairy products, combine 2.5 g product and 10.0 mL Diluent AP _____

b. Mix or knead until homogeneous, and add 5.0 mL to clean test tubes labeled "Blank" and "Test" _____

3. Add 0.1 mL MS water to the sample labeled "Blank", and 0.1 mL magnesium acetate solution to the sample labeled "Test" _____

4. Cap tubes and heat both aliquots for 1 hr at 34±1C _____

5. Remove samples from water bath and cool rapidly to 0-4.4C in an ice bath _____

6. Dilute 1 mL of sample containing magnesium acetate (Test) with 5 mL (1:6 dilution) of negative control product (item 11), label tube as "Diluted Test" _____

7. Test undiluted sample containing no magnesium acetate (Blank) and diluted sample containing magnesium acetate (Diluted Test) for phosphatase activity following item 15 (semisolid/solid dairy products begin at item 15b) _____

d. Interpretation _____

1. If the diluted aliquot containing magnesium acetate (Diluted Test) has equal (±30%) or greater phosphatase activity than the undiluted aliquot containing no magnesium (Blank), the sample is regarded as **Not Found** for residual phosphatase, and the phosphatase originally measured is of reactivated origin _____

   Dil. w/Mg (Test) Ó Undil. (Blank) = Reactivated

2. If the diluted aliquot (Diluted Test) contains less (30% below or less) activity than the undiluted aliquot (Blank) the sample is considered **Positive** for residual phosphatase _____

   Dil. w/Mg (Test) < Undil. (Blank) = Residual

3. A false-positive for residual phosphatase may also be obtained if a reactivatable sample has been allowed to stand at elevated temperatures (20C) for periods of 1 hr or more before testing (SPC < 20,000/mL) _____

## REPORT

**19. Report as:** _____

1. Residual phosphatase **Not Found (NF)**

   a. Report as < 44 mU/L (< 88 mU/L unflavored and flavored liquid dairy products or < 350 mU/kg solid/semisolid dairy products) _____

2. Residual phosphatase **Positive**

   a. Microbial and reactivatable phosphatase are not demonstrated _____

   b. Suspect positives greater than or equal (±30%) to 350 mU/L, category 4 or greater than the control point must be tested for microbial and reactivated phosphatase (items 16 and 17) _____

   c. Report mU/L values, mU/L range or greater than control point when equal to or greater than 44 mU/L fluid white milk, 88 mU/L unflavored and flavored liquid dairy products or 350 mU/kg semisolid/solid dairy products _____

3. Report as **Not Found** for residual phosphatase if: _____

   a. If microbial phosphatase present _____

   b. If reactivated phosphatase present _____

   c. If there is documentation to show that the product was treated such that reactivated phosphatase may be present _____